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Respiratory Neuron Activity in the Mesencephalon, Diencephalon and Cerebellum of the Carp

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Summary. The functional properties, localization and connections of neurons with a respiratory-rhythmic firing pattern in the mesencephalon, diencephalon and cerebellum of the carp were studied. Some neurons acquire respiratory rhythm only as a side effect of respiration via sensory stimulation by movements or water current. In other neurons, however, the rhythm is the result of nervous input from the respiratory center. These were studied in more detail. Three categories were distinguished:

1. Plain respiratory neurons. None of the stimuli used could influence the firing pattern of these neurons.
2. Respiratory-optical neurons, which receive both respiratory and visual input.
3. Respiratory-movement-sensitive neurons, the firing pattern of which is sensitive to stimuli influencing the respiratory movements.

The respiratory-optical neurons are argued to be components of a system correcting the visual image for respiration-induced displacements of the eyes. The movement sensitive neurons appear to process proprioceptive information at a level, where the demands on the cranial muscles of respiratory and other movements are integrated. The plain respiratory neurons, at least partly, can be regarded as interneurons between the respiratory center and the systems mentioned above.

Introduction

After electrophysiological observations (Adrian and Buytendijk, 1931; Woldring and Dirken, 1951) indicated the existence of respiratory neurons in the medulla oblongata of fishes and ablation experiments (Shelton, 1959) showed that those animals can generate fairly normal respiratory movements when all parts of the brain except the medulla oblongata are removed, all investigations concerning neural structures related to the respiratory system have been performed in that part of the brain (Ballintijn, 1972; Ballintijn and Alink, 1974, 1977; Bamford, 1974; Baumgarten and Salmoiraghi, 1962; Shelton, 1961; Shelton, 1970; Waldron, 1972).

In the course of our research on the proprioceptive regulation of respiratory muscle contraction in the carp (Ballintijn, 1972; Ballintijn and Bamford, 1975; Ballintijn and Roberts, 1976; Luiten, 1975; Luiten and Van der Pers, 1977; Luiten, 1979), however, the necessity arose to explore the mesencephalon. The reason was that in higher vertebrates primary proprioceptive signals from the jaw muscles are processed in the nucleus mesencephalicus trigemini. Such a nucleus, on comparative morphological grounds, is also regarded to be present in teleost fish and as their jaw muscles have an important respiratory function, it became necessary to determine whether this nucleus plays the same role as in higher vertebrates. For this purpose neuroanatomical experiments using the Horse Radish Peroxidase technique and electrophysiological recording experiments were performed. The HRP data clearly showed that in carp the mesencephalic trigeminal nucleus does not receive primary proprioceptive information from respiratory muscles. The neurophysiological experiments, however, quite unexpectedly lead to the discovery that in the mesencephalon and also in the cerebellum and diencephalon, many neurons are active in respiratory rhythm. This paper reports their properties, position and possible function.

Methods

Carp of 18–24 cm length were used in all experiments. They were anaesthetized in a Tricaine methane sulfonate (MS 222) solution

Abbreviation: HRP, horse radish peroxidase

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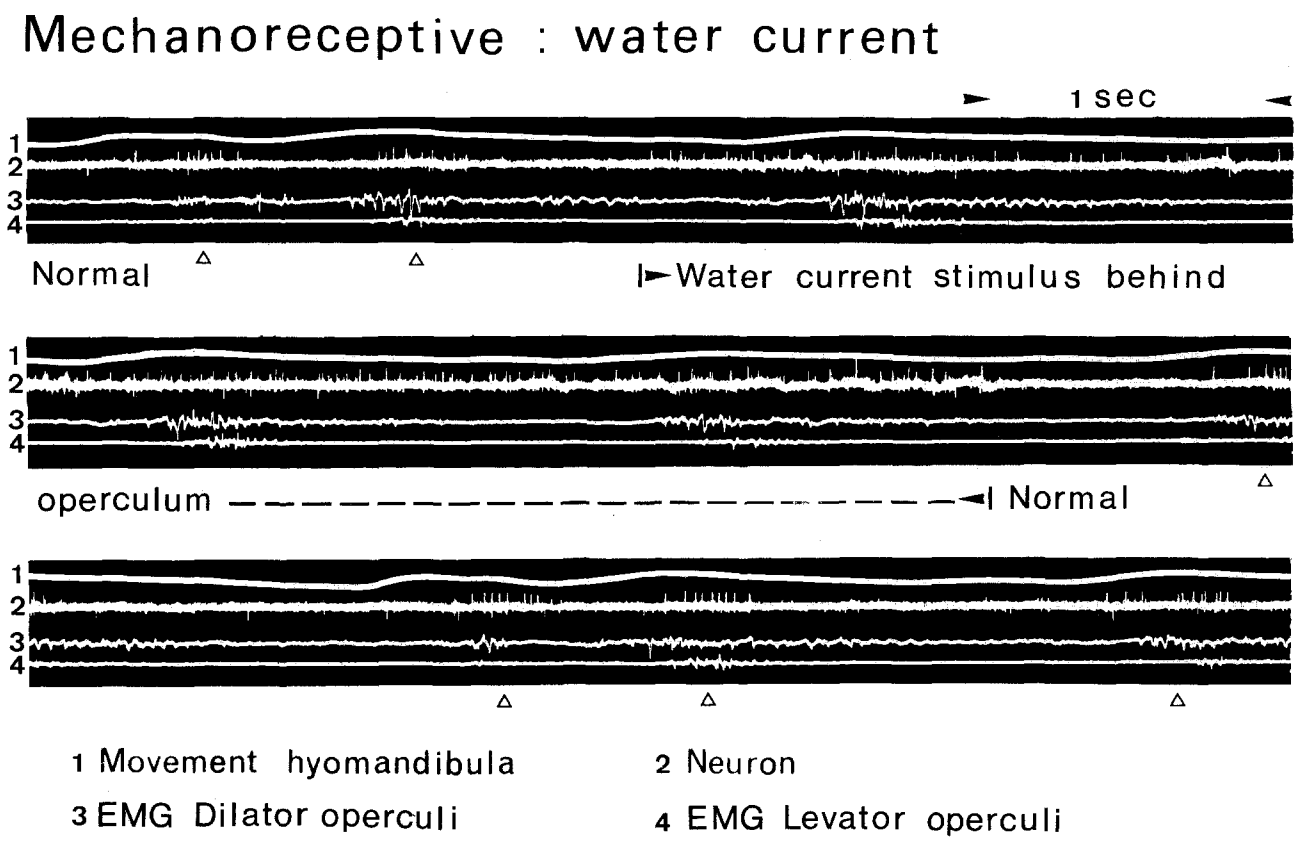

Fig. 1. Respiratory-rhythmic neuron activity generated as a side effect by the exhalant water current. Artificial stimulation with a continuous jet of water behind the operculum results in continuous activity

(30–50 mg/l) and fixed in a clamp. A hole was made in the skull, exposing the mesencephalon and cerebellum. The spinal cord was transsected just behind the skull. After that, the fish were transferred to an experimental tank filled with normal water without anaesthetic. The water level was kept just below the border of the hole in the skull, but the opercula were completely submersed. In this condition the fishes were breathing normally and generally could still move their pectoral fins, which made quiet swimming movements.

Neuron activity was recorded using either glass capillary or silver plated tungsten electrodes, both with tip sizes ranging between 1 and 6 µm. The capillary electrodes were filled with a 10% HRP solution in 0.6 M Tris HCL buffer which, after a successful recording, could be injected iontophoretically (1.25 µA during 30 min). To obtain neuroanatomical information about the connections of the recording site, the fishes were allowed to survive for ca. 10 days, after which the transport of HRP was studied in histological sections of the brain (Luiten, 1975). With the metal electrodes very small silver marks could be made enabling a more precise localization of the electrode tip position (von Baumgarten et al., 1960).

Electromyograms of the primary respiratory muscles were recorded with unipolar, varnish insulated stainless steel electrodes (diam. 150 µm) with exposed tips bevelled to an angle of about 60°.

The signals from the metal microelectrodes were amplified with Grass P15 amplifiers, those from the capillary electrodes with Grass P16 amplifiers. The upper frequency limit for the electromyograms was set at 3 kHz and for the neuron signals at 10 kHz.

All the data, including a movement record from the anterior border of an operculum (which represents hyomandibular movements) and stimulus markers were recorded on a Bell and Howell instrumentation tape recorder, together with experimenters comments on the voice track.

To investigate the functional properties of the neurons recorded, the following stimuli were used:

1. Total darkness;
2. Homogeneous illumination of the eyes;
3. Placing a screen behind the opercular slits to direct the exhaled water current away from the body;
4. Squirting a jet of water into the mouth;
5. Squirting a jet of water onto the body behind the opercula;
6. Forced abduction of the lower jaw or operculum;
7. Forced adduction of the lower jaw or operculum.

Results

In investigations on the medullary respiratory center, the fact that a neuron displays activity with a respiratory rhythm generally has been interpreted as an indication that it is part of – or is connected to – the respiratory control system. Although probably valid for medullary neurons, this criterion certainly can not be used in the mesencephalon, diencephalon and cerebellum. The reason is that in these parts of the brain neurons from a number of systems are present

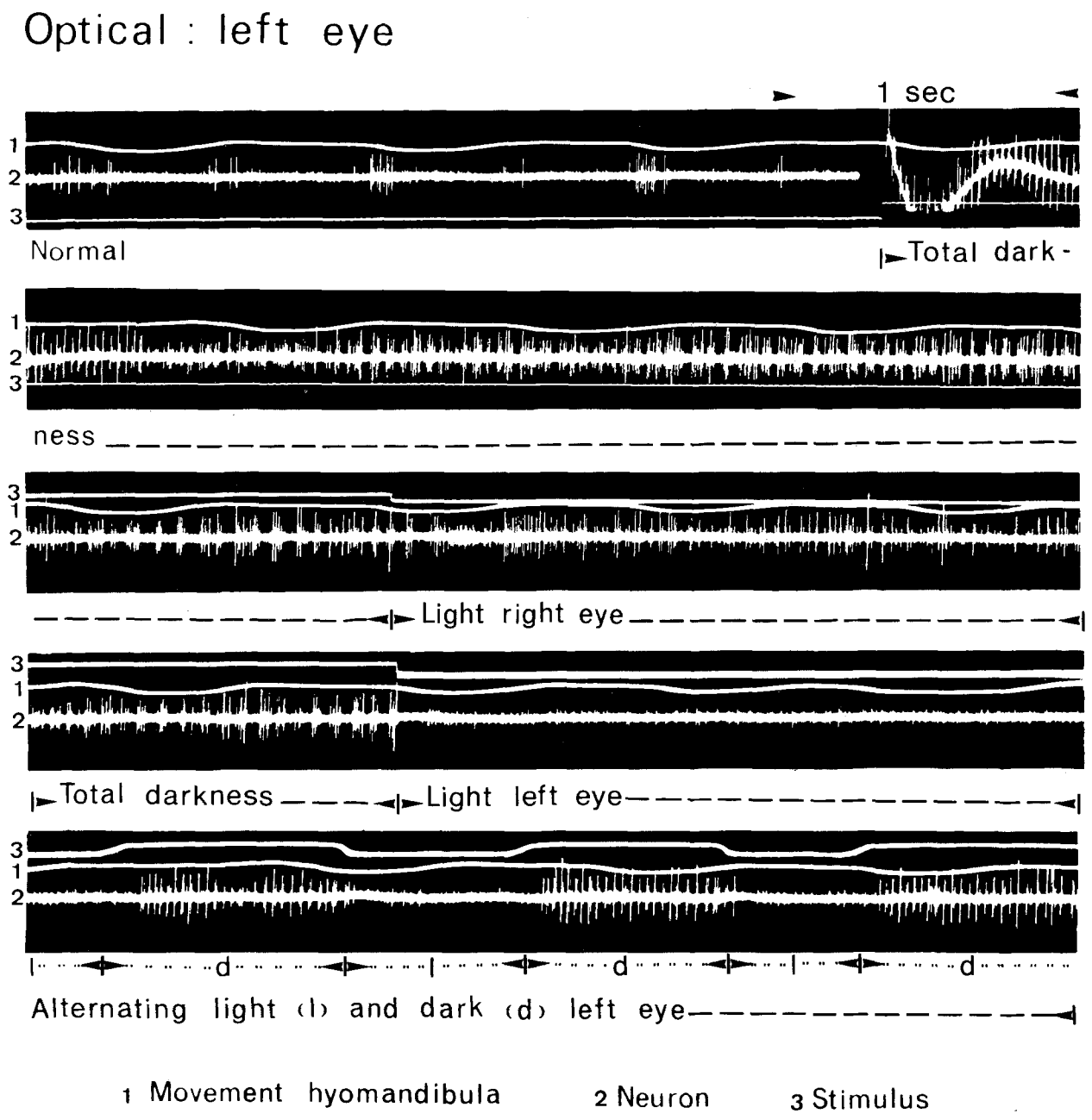

Fig. 2. Respiratory-rhythmic neuron activity with two bursts per respiratory cycle, generated as a side effect by respiration-induced movements of the left eye. (The existence of large and smaller spikes indicates the presence of neighbour neurons with the same firing characteristics.) In total darkness, or with only the right eye illuminated, the neurons become continuously active (the slight difference in activity being the result of stray light reaching the left eye). Homogeneous illumination of the left eye inhibits the activity completely while an alternating light-dark stimulus to the left eye generates a bursting firing pattern synchronous with the stimulus

which, via respiration modulated sensory input, acquire respiratory rhythmicity as a side effect. This is especially the case with:

1. neurons connected to the part of the water current perception system located behind the opercular slits, which is stimulated by the pulsating water flow leaving the opercular cavities;

2. neurons of water pressure receptors which may be present in the buccal and opercular cavities and also behind the opercular slits;

3. neurons belonging to the visual system, the activity of which is modulated in respiratory rhythm because the eye, being bordered on three sides by moving parts of the respiratory pumps, is forced to

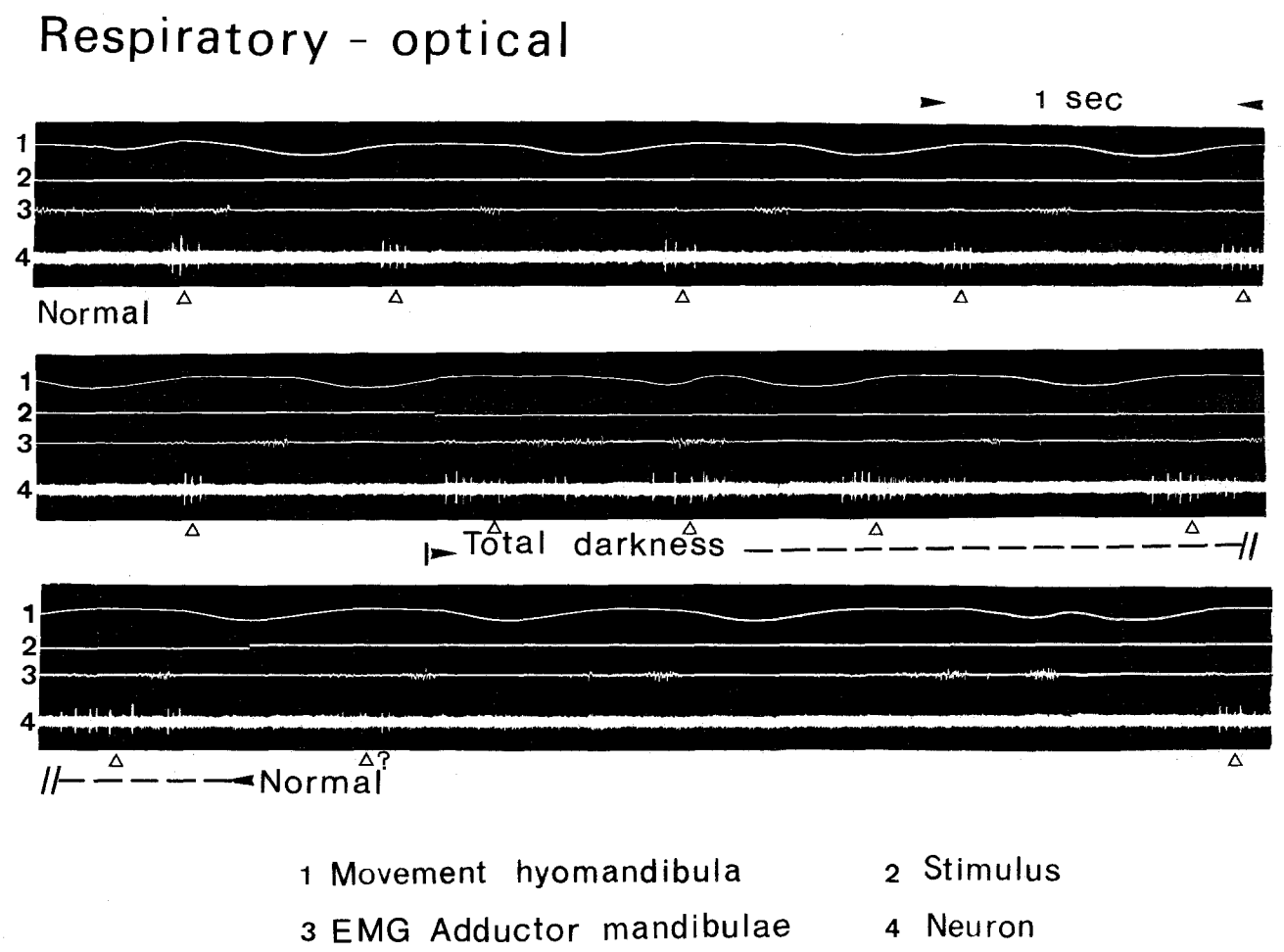

Fig. 3. Neuron maintaining its respiratory rhythmicity in total darkness. The burst duration then is increased and neighbour neurons recruited. Transition from dark to light results in inhibition during the first few seconds, after which the normal activity is resumed. The first burst after the transition (marked △?) because of the small spike amplitude can be ascribed to a neighbour neuron

make small rotational movements in phase with respiration which result in displacements of the image on the retina.

As in the present study our interest was focussed on neurons with a nervous connection with the respiratory control system, the first step in the experimental procedure always was to determine with a number of "standard tests", whether the respiratory rhythmic activity of a neuron under observation was due to respiratory center input or to one of the side effects mentioned above. This was done preferably by eliminating the side effect stimulus completely or else by giving continuous intensity artificial stimuli of the same nature as the suspected side effect (e.g. homogeneous illumination of the eye, constant water current). Under these circumstances the neuron under observation should retain its respiratory rhythmicity, in order to be classified as "connected to the respiratory system". It then was subjected to further analysis. The following "standard tests" were routinely performed:

Stimulating effects of the respiratory water current were abolished by placing screens behind the opercular slits, deflecting the water current from the body. A possible connection of the neuron studied with pressure or current receptors was further tested by generating water pressure pulses along the body of the fish behind the opercula and in the respiratory cavities. Figure 1 shows an example of a neuron from this category. Normally it fires in respiratory rhythm but when a water current is directed at the body behind the operculum it becomes continuously active.

Stimulation of optical system cells by movements of the image on the retina was prevented by putting the fish in total darkness or giving a homogeneous illumination to the eye. An example of a pure optical-system-neuron, modulated in respiratory rhythm, is

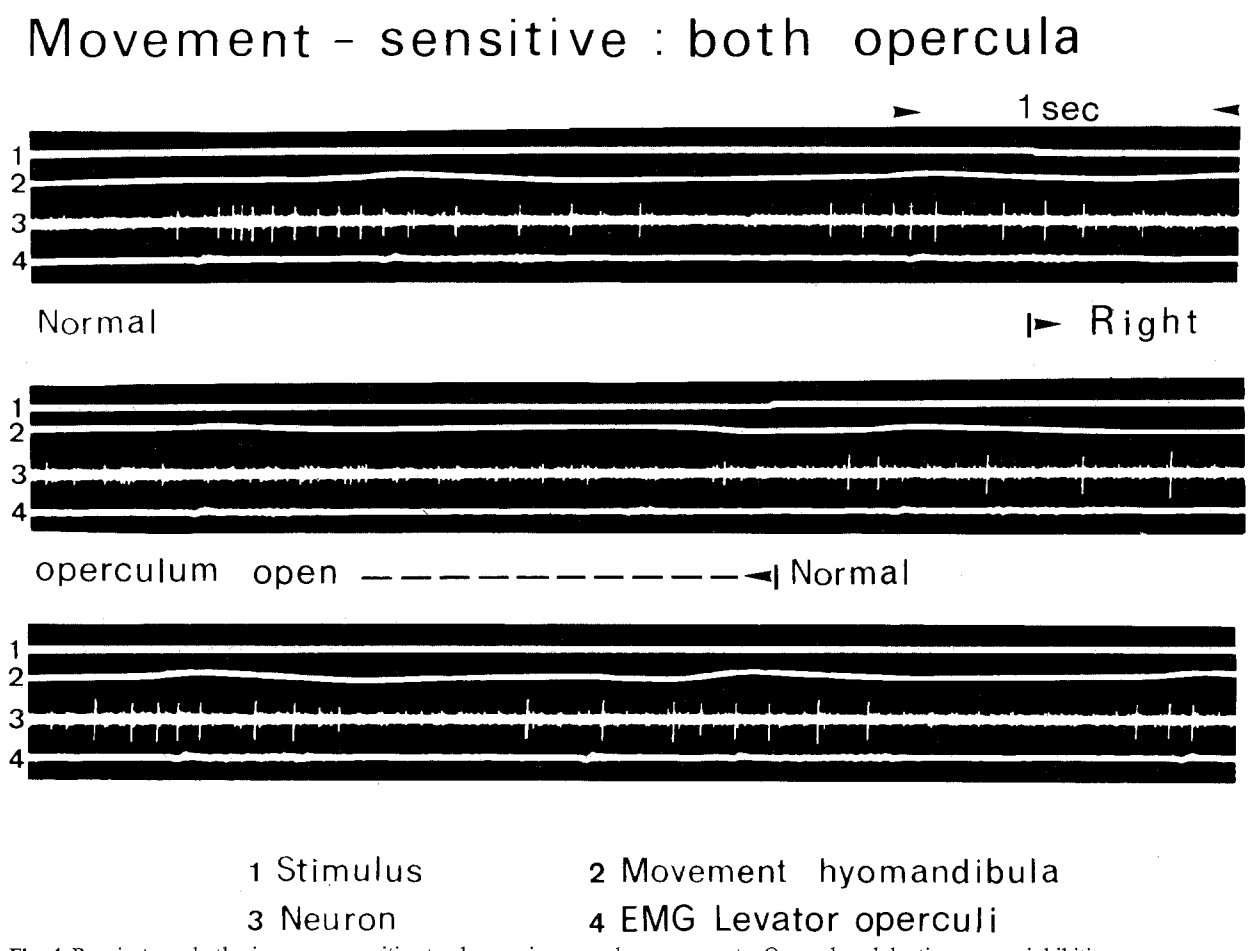

Fig. 4. Respiratory rhythmic neuron sensitive to changes in opercular movements. Opercular abduction causes inhibition

given in Fig. 2. Under normal light conditions two bursts of activity per respiratory cycle are present, one with a larger and the other with a smaller number of action potentials. Additional low-amplitude spikes indicate the existence of neighbouring neurons with the same activity characteristics. During total darkness, especially at the beginning of the stimulus, neighbour neurons seem to be activated as well, but all units recorded display continuous activity (with a high frequency modulation as described by O'Benar (1976) for some visual neurons) and no respiratory frequency modulation can be detected. Homogeneous illumination of the right eye does not cause a marked change but the same stimulus presented to the left eye abolishes all neuron activity (the small effect during illumination of the right eye probably being due to stray light reaching the left eye). This indicates that the input of the neuron under observation is derived from the left eye. This conclusion is confirmed by the observation that an alternating light-dark stimulation of the left eye generates a bursting firing pattern, synchronous with the stimulus instead of with respiration.

The neurons of which the respiratory rhythmicity on the grounds of their reaction to the "standard tests" thus was shown not to be caused by side effects but by a neural connection with the respiratory system, were subjected to a further analysis. They can, at present, be divided into three different categories and will be called "respiratory neurons" in the following.

1. Plain Respiratory Neurons

The first category of neurons (of which 6 have been found in various places) is called plain respiratory neurons, because after their respiratory nature was established with the "standard tests", no stimulus influencing the respiratory system (e.g. forced artificial movements of the respiratory pumps by manipulating the lower jaw or opercula) could influence their firing pattern. Horseradish peroxidase marks, deposited following recording, were present in the dorsal aspects of the mesencephalic tegmentum close to the posterior commissure (Fig. 6B). As a result

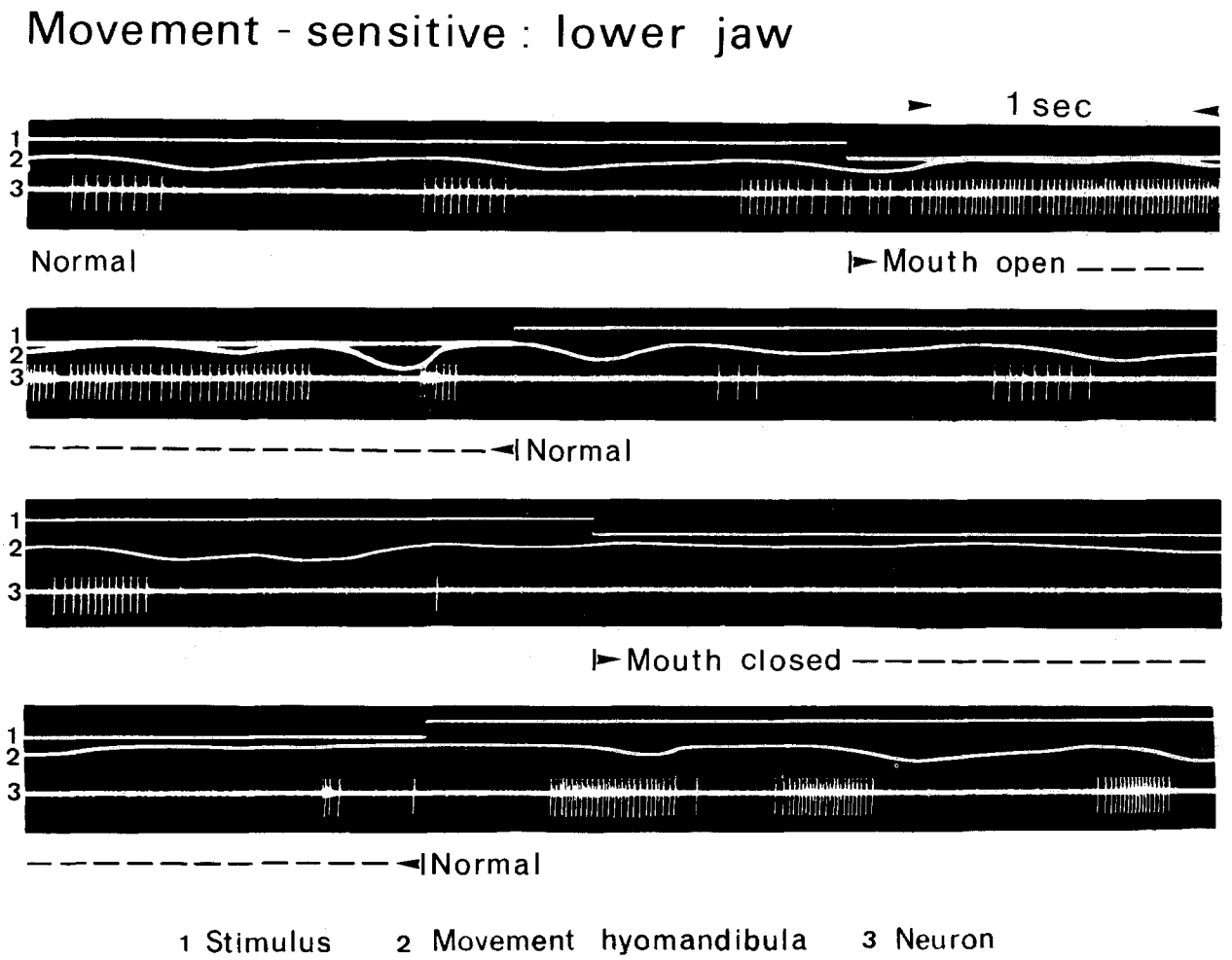

Fig. 5. Respiratory-rhythmic neuron sensitive to lower jaw movements. Abduction of the jaw results in continuous firing, adduction in complete inhibition. Other movement stimuli (e.g. operculum) have no effect

of HRP transport, bilateral fiber connections running in this commissure could be detected and projections to the tectum opticum and the nucleus rotundus were also found.

2. *Respiratory-Optical Neurons*

The second category (6 respiratory rhythmic neurons) respond to optical stimuli. They maintain their respiratory rhythm, however, in total darkness or with homogeneously illuminated retina.

Therefore the conclusion must be drawn that this rhythmicity is not due to displacements of the retinal image, but is a result of neural signals from the respiratory control system. Thus these neurons apparently receive both respiratory and optical information. An example of this category is given in Fig. 3.

In total darkness the neuron retains its respiratory rhythm all the time. An additional optical input is, however, indicated by the facts that *a:* its burst length is markedly increased during the dark period when neighbour neurons with spikes of lower amplitude seem to be recruited as well, and *b:* after the transition from dark to normal environmental light it becomes completely silent for about 3 respiratory cycles or 2.5 s before it resumes its normal respiratory activity.

The horseradish peroxidase marks of the neurons in this category were found in the stratum griseum et fibrosum of the most rostral aspects of the tectum opticum (Fig. 6A). From the HRP injection spots, anterograde labeling occurred in the commissura horizontalis, which projects to the nucleus rotundus and nucleus glomerulosus and in the tractus tecto-bulbaris projecting to the mesencephalic and bulbar reticular formation. Retrograde labeling was observed in pretectal nuclei, nucleus isthmi and torus semicircularis. A more detailed description of these connections is given elsewhere (Luiten, in prep).

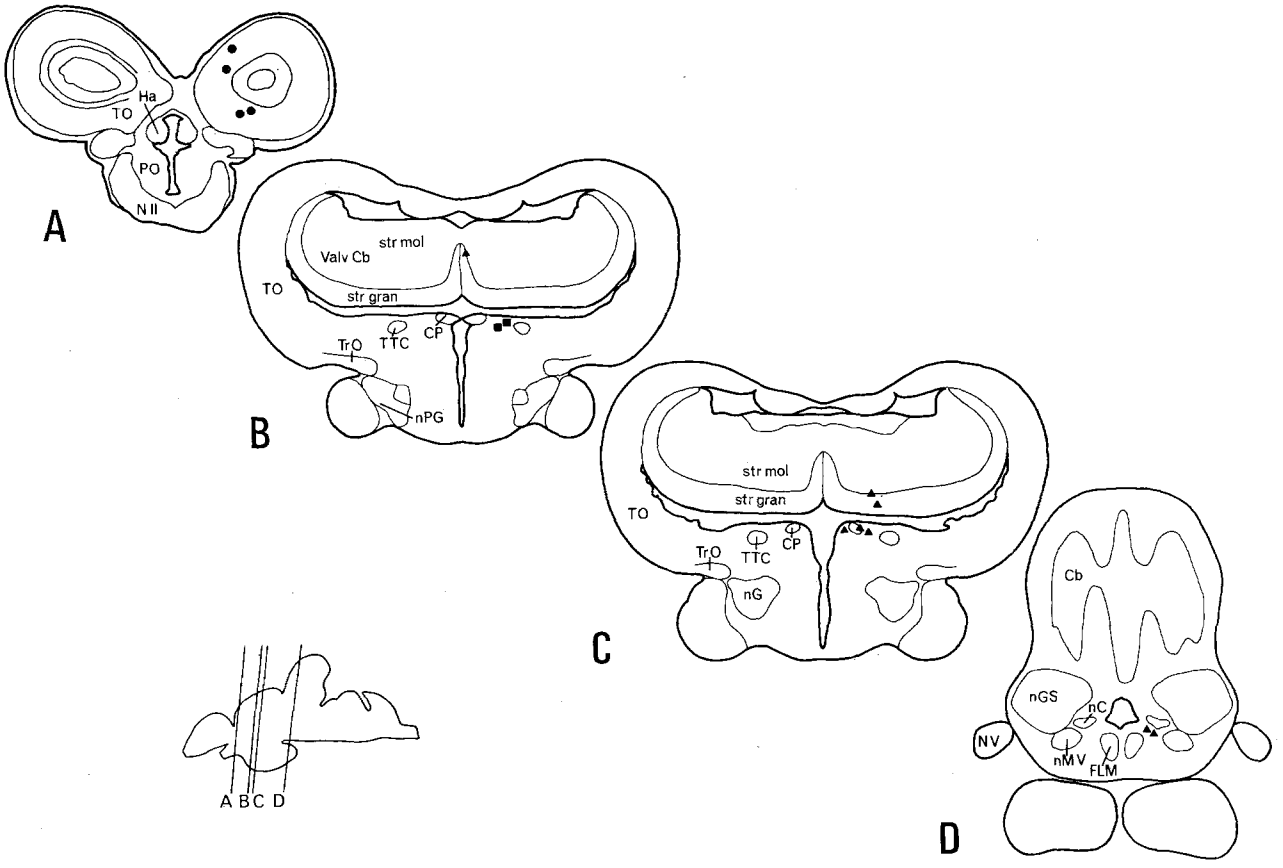

Fig. 6A–D. Series of transverse sections from rostral (**A**) to caudal (**D**) with the recording sites of neurons described in this paper as determined with iontophoretic horseradish peroxidase injections. Triangles: respiratory movement sensitive neurons. Squares: plain respiratory neurons. Rounds: respiratory-optical neurons. *Cb*, Corpus cerebelli; *CP*, commissura posterior; *FLM*, fasciculus longitudinalis medialis; *Ha*, nucleus habenularis; *NC*, nucleus cerebelli; *NG*, nucleus glomerulosus; *nGS*, nucleus gustatorius secundus; *nMV*, nucleus motorius nervi trigemini; *nPG*, nucleus preglomerulosus; *N II*, nervus opticus; *NV*, nervus trigeminus; *PO*, nucleus preopticus; *str gran*, stratum granulosum; *str mol*, stratum moleculare; *To*, tectum opticum; *TrO*, tractus opticus; *TTC*, tractus tectocerebellaris; *Valv Cb*, valvulae cerebelli

3. Neurons Responding to Changes in Respiratory Movements

A third category of 13 neurons, which on the ground of the "standard tests" can be classified as "respiratory neurons", responds to changes in the respiratory movements. They can be stimulated by mechanical loading of the buccal or the opercular system. Their reaction to such stimuli may be different. Some neurons react to impeding the abduction of the lower jaw or opercula, others when the adduction is hindered and still others respond to both stimuli. The neuron of Fig. 4, for instance, is influenced by opercular abduction. It is then silenced completely. It appears to have bilateral connections because it responds in the same way to manipulation of either operculum. Other neurons with unilateral opercular input also exist. The neuron of Fig. 5 responds to both abduction and adduction stimuli applied to the lower jaw. The response to abduction is continuous firing and to adduction complete inactivity.

Electrode marks in this group of recordings were found in the principal sensory trigeminal nucleus (Fig. 6D), in the granular layer of the valvulae cerebelli and in the nucleus of the posterior commissure (Fig. 6B,C). Injections of HRP at the cerebellar recording sites (after a suitable survival time) resulted in retrograde labeling in the principal sensory trigeminal nucleus and in the nucleus lateralis valvulae. Reciprocally the HRP injections in the nucleus princeps V were followed by anterograde labeling of fibers, projecting to the granular layer of the valvulae cerebelli. Injections in the posterior commissure gave rise to anterograde fiber marking in the most rostral parts of the torus longitudinalis.

Discussion

The existence of neurons which receive respiratory and optic signals as described in the previous section, suggests an interaction between the respiratory and the visual system. Interactions between other systems in the brain of fishes have been reported in the literature.

Page and Sutterlin (1970) found neurons in the midbrain tegmentum which reacted to both auditory and visual stimuli and O'Benar (1976) reports the convergence of tactile or acoustic with visual input in some tectal neurons. Further Allum et al. (1976) described the interaction of visual and vestibular signals in the vestibular nuclei of the goldfish. This interaction leads to an improvement of the response of the vestibular system to low frequency movements. It especially enhances the accuracy with which the fish can determine its position in space during slow movements.

Johnstone and Mark (1969) and Hermann (1971) provided evidence for the correction of visual system signals during saccadic eye movements of fish. The first mentioned authors found units in the tectal commissure with an activity pattern in phase with, but starting before the eye movements. These units were not of a sensory nature, because their activity was not affected by tectal ablation or paralysis of the fish and also did not belong to the motor system. They are regarded as interneurons, cancelling the burst of afferent optical activity caused by the eye movement. In this way perceptual stability is thought to be attained during the eye movement. Hermann (1971) reports on saccade-correlated potentials in the tectum and cerebellum which partly also precede the movements. He concludes that their function is to register the saccade with the eye-body postural coordinating systems of the cerebellum.

It is not surprising that in fish optical data play an important role in the neural systems processing movement and postural information. In quadrupeds, ground contact is an important source of reference which in fish, floating in the water, lacks completely. The latter, in fact, can only obtain direct information on the stable elements in their environment, and thus on their own position, through visual observation. In this connection adequate correction of visual data for disturbing influences seems extremely important. At the beginning of the results section it was already mentioned that the respiratory movements cause small rotations of the eyes. This, of course, results in displacements of the image on the retina, which in themselves cannot be distinguished from movements of the fish in the water. A correction of these disturbances can only be obtained if the visual system receives information about the ongoing respiratory movements. Such information is present in the activity of the respiratory-optic neurons we found in the area of the nucleus rotundus and in the tectum. These neurons receive respiratory signals, not as a side effect, but from the respiratory system itself and process this information together with input from the visual system. Thus, in our opinion, there are strong indications that they are elements of a system correcting the visual image for respiratory displacements of the eye, analogous to the neurons described by Johnstone and Mark (1969) that cancel optical activity due to saccadic eye movements.

The respiratory-optic neurons must obtain their respiratory rhythmic input ultimately from the respiratory center. It is possible that some members of the group of neurons called plain respiratory neurons, which did not react to any stimulus given in the present experiments, play an intermediate role and are, in fact, interneurons connecting the medullary respiratory system to the respiratory-optic neurons. The fact that after peroxidase injections near plain respiratory neurons labeling was found in the tectum opticum and nucleus rotundus agrees well with this view.

Proprioceptive signals, as was concluded in earlier papers in which the effect of short experimental muscle twitches upon the activity of respiratory neurons in the medulla oblongata was studied, besides playing a role in direct proprioceptive muscle control reflexes, are also processed at higher levels of organization (Ballintijn and Alink, 1977; Ballintijn and Bamford, 1975; Ballintijn and Roberts, 1976). There, these mechanoreceptive signals seemed to contribute to long-term respiratory events. Apparently the respiratory-movement-sensitive neurons, recorded from in the present experiments, are part of such a system. This view is further justified by the fact that the structures in which they are located maintain direct connections with the descending trigeminal nucleus which contains second order neurons receiving the proprioceptive signals from the respiratory muscles (Luiten, 1975).

The localization of the respiratory-movement-sensitive neurons in the cerebellum itself or in structures that are closely related to the cerebellum, which is generally regarded as the ultimate movement coordinating system, is of further interest. It seems to indicate that they play a role in the integration of all the movement demands, including those of respiration, imposed upon the cranial muscles. This conclusion agrees well with the results Kotchabhakdi (1976) obtained on the goldfish, where various stimuli where shown to impinge upon individual Purkinje cells, in which they appeared to elicit similar activity patterns.

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